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LOCALIZATION OF SPECIFIC mRNAs FOR HUMAN PLACENTAL LACTOGEN AND HUMAN CHORIONIC GONADOTROPIN-ALPHA AND BETA SUBUNITS

A Thesis

Presented to the

Faculty of

California State

College, San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

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by

Richard Fehn

May 1978

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Dean

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ABSTRACT

Total RNA was extracted from term human placentae obtained from cesarean sections. The total mRNA fraction was isolated and fractionated over a sucrose density gradient which yielded 12S and 18S peaks. Individual fractions from across the profile were translated in a cell-free, rabbit reticulocyte translation system and only the 12S region was found to contain messages for hPL and hCG as determined by immunologic and autoradiographic techniques. Acrylamide gel electrophoresis of the 12S region revealed at least three species of RNAs of approximately 166,000 to 224,000 molecular weight (size estimates based upon methylmercury gels). Molecular weights of the synthesized proteins closely approximated those of hPL, hCG-alpha and hCG-beta subunits as described in current literature. The relative quantities of specific synthesized proteins appear to be correlated with the relative amounts of specific RNA species in the 12S region.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Throughout gestation, the human placenta functions in an endocrine role by producing both steroid and protein hormones which influence both fetal and maternal physiologies (Josimovich et al., 1974). Although both classes of hormones are actively synthesized by the placenta, only the protein hormones will be considered in this study.

The two major protein hormones secreted by the human placenta are placental lactogen (hPL) and chorionic gonadotropin (hCG). Human placental lactogen is a simple polypeptide containing 191 amino acids and has an estimated molecular weight of 22,500 daltons (Niall, 1971). Produced by the syncytiotrophoblast (Sciarra et al., 1963), it is thought to be synthesized as a larger pro-hormone (Boime et al., 1975; Cox et al., 1976; Seeburg et al., 1977), which undergoes a membrane-dependent cleavage followed by packaging and storage in its native form. Unlike hPL, hCG is a complex glycoprotein of about 30,000 molecular weight and is composed of two subunits, alpha and beta (Bahl et al., 1972). The alpha subunit is very similar or identical to the alpha subunits of glycoprotein hormones from the anterior pituitary (e.g., follicle stimulating hormone, luteinizing hormone and thyroid stimulating hormone), while the beta subunit possesses the biologically active sites and

immunologic determinants of the intact hormone (Turner and Bagnara, 1976). Conflicting reports indicate that hCG may be produced by either the syncytiotrophoblast, cytotrophoblast, or both (Chung et al., 1969; Loke et al., 1972). In addition, Yoshimoto et al. (1977) have found the production of beta chain hCG in several nonendocrine tissues. However, only the protein portion has been demonstrated and it retains little or no biological activity.

Serum levels of these two hormones vary as a function of gestational age, hCG being highest during the first trimester and decreasing towards term, and hPL having its lowest concentration during the first trimester and increasing through parturition (Josimovich et al., 1974). In addition, the term placenta synthesizes an estimated 1 to 2 grams of hPL per day, which is approximately 80 per cent of all secreted proteins during that period (Suwa and Friesen, 1969).

The placenta is an interesting transitory endocrine gland and a valuable tissue for studying the regulation of hormone production and control of protein synthesis in general. Elevated rates of hPL synthesis combined with the possibility of feedback circuits in hPL-hCG production provide a nearly ideal arrangement for such regulatory studies. However, before the mechanisms controlling synthesis can be elucidated the following processes must be

understood (Gusseck, 1977):

1. The rate of hormone release relative to the intracellular steady state level.

2. The rate of intracellular degradation relative to synthesis of the hormone molecule (turnover rate).

3. The rate of hormone-specific messenger RNA translation.

4. The rate of hormone-specific mRNA turnover.

5. The rate of hormone-specific mRNA synthesis.

The last three processes require that a hormone-specific mRNA be identified and isolated to permit its manipulation within the given parameters.

The intent of this study is to localize and identify the specific mRNAs for hPL and hCG alpha and beta subunits from human placentae. Messenger-RNAs for these hormones should be contained within the bulk RNA extracted from placentae. Further isolation should reveal that these messages are polyadenylated as are most active mRNAs. The molecular weights of these messages are expected to be lower than most mRNAs because they code for low molecular weight proteins. Isolation techniques developed herein should be useful for subsequent experiments to determine mechanisms of mediating peptide hormone production.

METHODS AND MATERIALS

Total RNA Preparation

Term human placentae obtained from cesarean sections were transported on ice to the laboratory where processing was begun within one hour. Two different methods of isolating bulk ribonucleic acids (RNA) were used (Figs. 1 and 2) to demonstrate that the final RNA product was not particular to a specific extraction technique. These procedures were used interchangeably as the resultant RNA was the same, regardless of which technique was used.

In the first method (Chirgwin et al., 1977), 40 grams of tissue were homogenized in 200 ml of a buffer which consisted of 7 M guanidinium thiocyanate (Alfa Chemicals), 0.1 M Mercaptoethanol (MSH), 50 mM Tris, 50 mM Ethylenediamine tetracetate (EDTA) at a pH of 7.0 using a Brinkman Polytron. Following a 10 minute centrifugation at 10,000 times gravity (xg), the volume of the membrane-free supernatant was measured and cesium chloride (Sigma) was added at a concentration of 0.2 g/ml of supernatant. The supernatant-cesium chloride mix was layered over a 6.0 ml pad of 5.7 M cesium chloride (CsCl), 50 mM EDTA at a pH of 7.0 in 1" x 3¼" nitrocellulose tubes and centrifuged in a SW-27 rotor at 25,000 rpm. for 45 hours at 15°C using a Beckman Model L-2 ultracentrifuge. Each tube was inverted to expose a clear

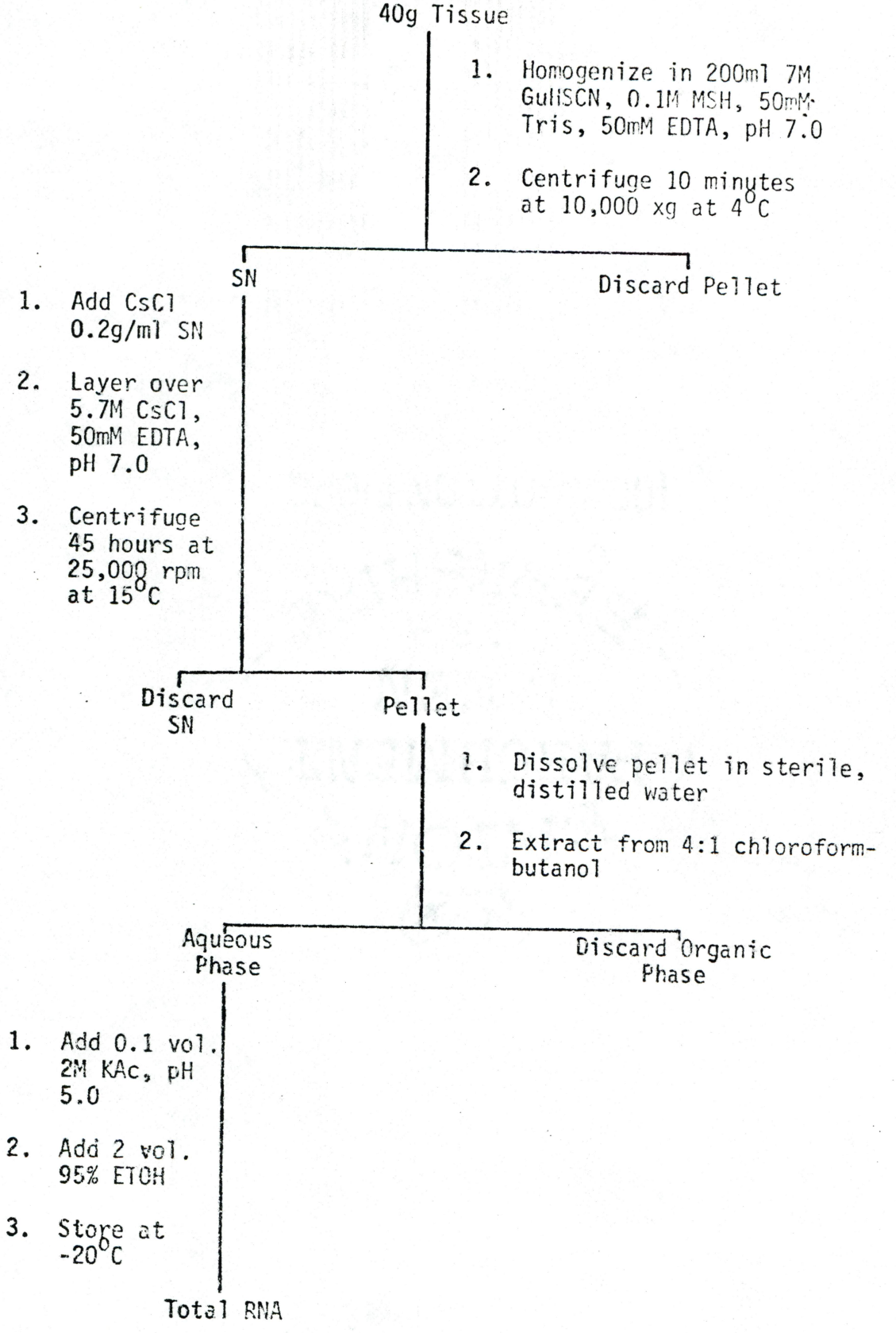

Fig. 1. Schema for the guanidinium thiocyanate isolation of total RNA.

RNA pellet and, after draining, the bottom one inch was cut off the tube using a razor blade. The pellets were dissolved in sterile distilled water and extracted from 8.0 ml 4:1 Chloroform-Butanol (water saturated). After measuring the volume of extract, a one-tenth volume of 2 M potassium acetate, pH 5.0, was added to the aqueous phase, followed by two volumes 95% ethanol (ETOH). The aqueous phase-ETOH mixture was stored at -20°C overnight to allow RNA to precipitate.

In the second method, a phenol-chloroform procedure was followed to extract total RNA. The soft tissues were dissected free from the chorioallantoic and amnionic membranes, cut into small sections, and then washed with cold saline. After draining the tissue, it was homogenized in two volumes of buffer (50 mM Tris-HCl, pH 7.4, 25 mM sodium chloride (NaCl), 5 mM magnesium chloride ($MgCl_2$), 250 mM sucrose, 0.5 mg/ml heparin) by three five-second bursts in a Waring Blender. The homogenate was centrifuged 10 minutes at 12,000 xg at 4°C. The supernatant was decanted and combined with one-tenth volume of 1% deoxycholate (DOC), 1% Triton X-100. The detergent-treated mixture was adjusted to 25 mM Tris, pH 7.4, 5 mM EDTA, 1% sodium dodecylsulfate (SDS), after which it was extracted with an equal volume of 1:1 phenol-chloroform. The solution was stirred for 20

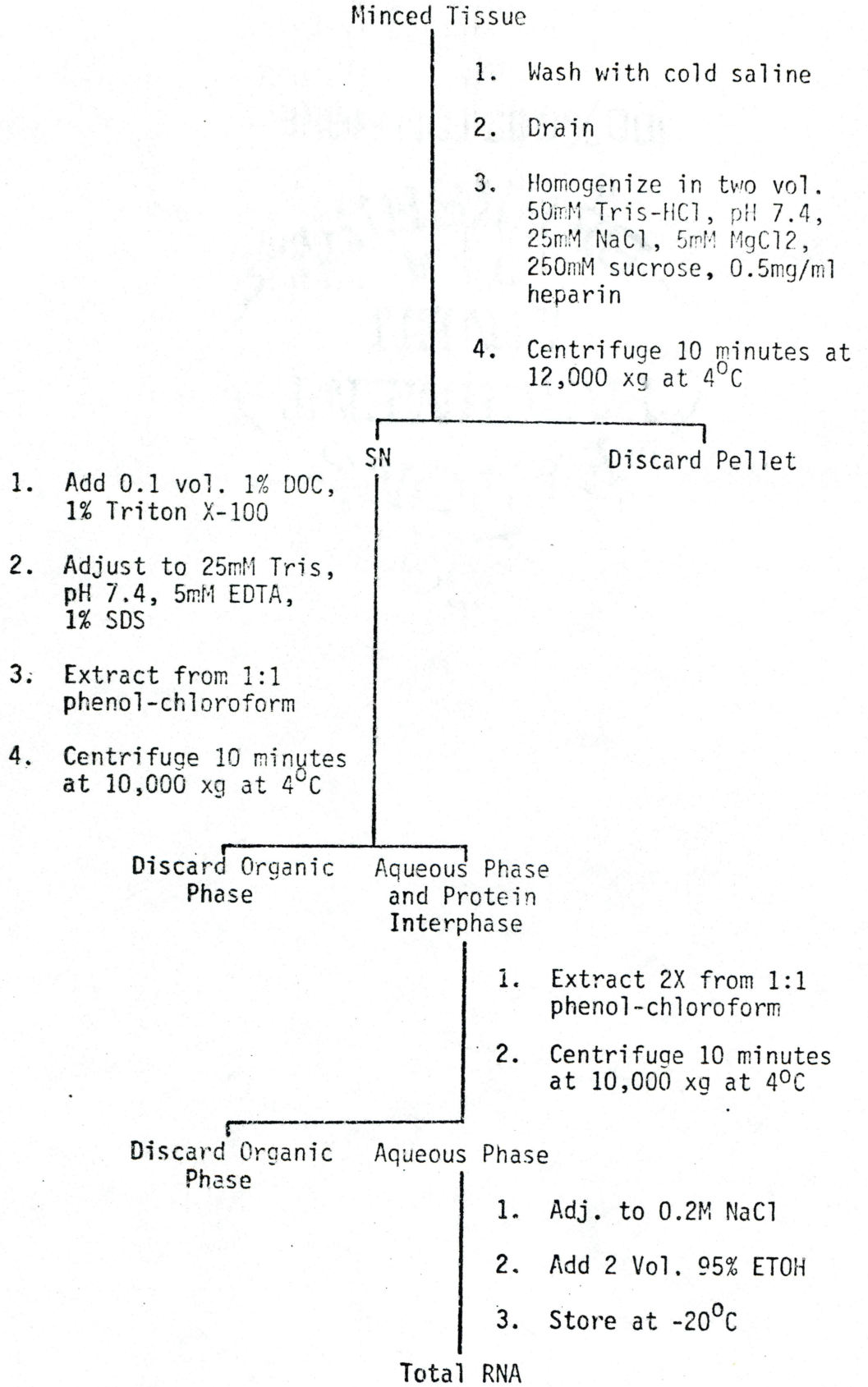

Fig. 2. Schema for the phenol-chloroform isolation of total RNA.

minutes at room temperature and then centrifuged 10 minutes at 10,000 xg at 4°C. The combined aqueous layer and protein interphase was removed and extracted two additional times with equal volumes of phenol-chloroform. After adjusting the aqueous phase to 0.2 M NaCl, two volumes of cold 95% ETOH were added to the mixture which was then stored at -20°C to precipitate the RNA.

mRNA Isolation

Bulk RNA from either of the above procedures was sedimented by centrifugation for 10 minutes at 10,000 xg at 4°C and prepared for oligo-(dT)-cellulose chromatography to extract the messenger RNA fraction by the method of Cabada et al. (1977). The pellet was resuspended in binding buffer (0.4 M NaCl, 1 mM EDTA, 0.1% SDS, 10 mM Tris-HCl, pH 7.5) and passed twice over a pre-equilibrated 1.0 ml bed oligo-(dT)-cellulose column (T-3 grade, Collaborative Research). Two 2.0 ml volumes of binding buffer were used to rinse the column followed by three 1.0 ml volumes of elution buffer (1.0 mM EDTA, 0.1% SDS, 10 mM Tris-HCl, pH 7.5). Three milliliters of eluate were collected, adjusted to 0.4 M NaCl and reapplied to the column. Two milliliters of low salt elution buffer (0.15 M NaCl, 1.0 mM EDTA, 0.1% SDS, 10 mM Tris-HCl, pH 7.5) were passed over the column followed by three 1.0 ml volumes of the original elution

buffers. These final three fractions were collected and adjusted to 0.1 M NaCl and precipitated from two volumes of 95% ETOH at -20°C.

Fractionation of mRNA

Messenger RNA, sedimented by centrifugation, was resuspended in sterile distilled water and layered over 5 to 20% sucrose gradients in polyallomer tubes. The resuspended RNA samples were centrifuged for three hours at 65,000 rpm. in a Beckman Model L-5 ultracentrifuge (SW-65 rotor). In order to determine the distribution of RNA in the gradients, each gradient was pumped through a UV monitor (ISCO, Model U-5) where absorbance at 260 nm (the wave length absorbed by nucleic acids) was checked to detect RNA. Various regions of the gradient were collected as individual fractions which were then precipitated from ethanol as before.

In vitro Translation of mRNA

Reticulocyte lysate was prepared from rabbits which had been made anemic by repeated injections of phenylhydrazine. This and subsequent processing was performed according to the method of Allen and Schweet (1962). Lysates were stored at -80°C until they could be treated with nuclease (Pelham and Jackson, 1976) to remove endogenous RNA. The treated lysates also were stored at -80°C.

In vitro translation was performed according to the assay of Eggitt et al. (1977). RNA samples (in distilled water) were added to 100 µl final volume reaction mixes which contained the following: 12 mM creatine phosphate, 4 µg creatine phosphokinase, 20 µM Hemin, 1.0 µCi (^{35}S) L-methionine (709 Ci/mmol, NEN), 50 µl nuclease-treated reticulocyte lysate, 1.0 mM ATP, 0.2 mM GTP, 75 mM KCl, 2.0 mM $MgAc_2$, 10 mM Tris-HCl, pH 7.6, 200 µM 19 L-amino acids minus L-methionine and RNA with a water balance volume where necessary. Incubation was for one hour at 37°C.

Total protein synthesis was determined by spotting 5.0 µl of reaction mix on Whatman GF/A discs which were subsequently washed as follows: 10% trichloroacetic acid (TCA), two ten-minute washes (2 x 10 minutes), 5% TCA 2 x 5 minutes, 5% TCA at 100°C for 15 minutes, 5% TCA rinse 2 x 1 minute. These discs were dried under a heat lamp and placed in individual counting vials containing 5.0 ml omnifluor (NEN). The radioactive content of each sample was quantitated by liquid scintillation spectrometry.

Evaluation of Synthesized Products

Gel electrophoretic, autoradiographic and immunologic techniques were used to identify the products of translation. Reaction mixes, each supplemented with an equal volume of detergent (0.5% DOC, 0.1% SDS, 1% Triton, 10 mM potassium

phosphate at a pH of 7.4), were combined with an equal volume of twofold concentrated sample buffer (Laemmli, 1970) and boiled for five minutes. The heat-treated mixtures were then cooled in an ice bath to prepare them for acrylamide gel electrophoresis. These treated samples were subjected to electrophoresis at a current of 15 ma through a 5% stacking gel and at 30 ma through a 12.5% separation gel as described by Laemmli (1970). Gels were stained for one hour in Coomassie Brilliant Blue with subsequent destaining in 50% methanol-10% acetic acid clearing solution.

Gels to be autoradiographed were treated as described by Bonner and Laskey (1974) by soaking in Dimethyl Sulfoxide (DMSO) 2 x 30 minutes, 20% Diphenyloxazole (PPO) in DMSO three hours, distilled water one hour and drying. Films for autoradiography (RP/X-Omat, Kodak) were placed in direct contact with PPO-treated gels and "sandwiched" between a sheet of plexiglass and a sheet of wood. The "sandwich" was placed in a light-proof box and exposed at -80°C.

A radioimmunoassay (RIA) for hPL was performed by adding 0.1 ml of reaction mix to 0.3 ml buffer (10 mM Tris-acetate, 1% bovine serum albumin), 0.1 ml diluted sheep anti-hPL antiserum and 0.1 ml ^{125}I-hPL (0.4 ng at 30 μCi/μg, New England Nuclear). Incubation of the reaction mix with the first antibody was for one hour at 37°C. An equal volume of 26% polyethylene glycol was then added as second

antibody and the mix was centrifuged one minute at 3,000 xg. The supernatants were aspirated and discarded following centrifugation. The remaining pellets were counted in a Nuclear Chicago gamma counter. A commercial RIA (Serano Labs) was used for quantitating the hCG-beta subunit.

Synthesized products were immunoprecipitated from 0.1 ml reaction mix by first adding 0.1 ml sheep antiserum plus 30 µl buffer (10% Triton X-100, 10% DOC, 10 mM Sodium phosphate, 150 mM NaCl at a pH of 7.5) and then incubating one hour at 37°C. Rabbit anti-sheep immunoglobulin G (0.1 ml) was added to the individual vials which were then incubated one hour at 37°C followed by storage at 4°C overnight. The immunoprecipitation mixes were vortexed and layered over a 1 M sucrose pad and centrifuged five minutes at 3,000 xg. The tubes were then frozen. Tube tips containing the precipitate were cut off, placed in individual counting vials containing 0.7 ml NCS solubilizer (Amersham) and dissolved for three hours at 37°C with shaking. Omnifluor was added to the vials which were assayed for radioactive content by liquid scintillation spectrometry following equilibration overnight.

Analysis of RNA

Individual RNA classes were separated according to their sedimentation coefficients in Svedberg units (S) to

approximate their sizes. Molecular weights of isolated RNAs were determined on 5 mM methylmercury-1% agarose slab gels (Bailey and Davidson, 1975). RNA samples were subjected to electrophoresis for 4.5 hours at 17.5 ma, stained 30 minutes in ethidium bromide and viewed on a short wave UV transilluminator. Gels were photographed through a yellow filter using Kodak Tri-X pan film. Ultraviolet absorbance scans were performed on an ISCO tube gel scanner using RNA samples run on 3% acrylamide gels for three hours at 5 ma per tube. Quartz tubes were used to permit monitoring of absorbance at 260 nm to locate RNA bands.

RESULTS

Localization of hPL mRNA

The size class of RNA responsible for the synthesis of hPL was determined by *in vitro* translation of various regions of the total RNA profile which were fractionated over a sucrose density gradient (Fig. 3,a). Production of hPL was assayed by RIA. No hPL synthesis was directed by RNA from either the 4-5S peak (tRNA) or 28S peak (rRNA) as expected. The 18S peak, which contains considerable amounts of mRNA in addition to rRNA, also showed no hPL synthesis. Only in the area of the small 12S peak was hPL produced (Fig. 3,b).

RNA Characterization

Further purification of the RNA by oligo-(dT)-cellulose chromatography showed that the 12S peak was polyadenylated (poly A). A sucrose gradient profile of this poly (A) RNA (Fig. 4,a) showed that the 12S peak was retained along with the mRNA-containing 18S peak. The 12S peak was isolated and analyzed by sucrose density centrifugation. The sedimentation coefficient was 12S as before (Fig. 4,b) demonstrating the intactness and stability of this RNA.

Analysis of the 12S poly (A) RNA peak by acrylamide gel electrophoresis revealed at least three species of RNA

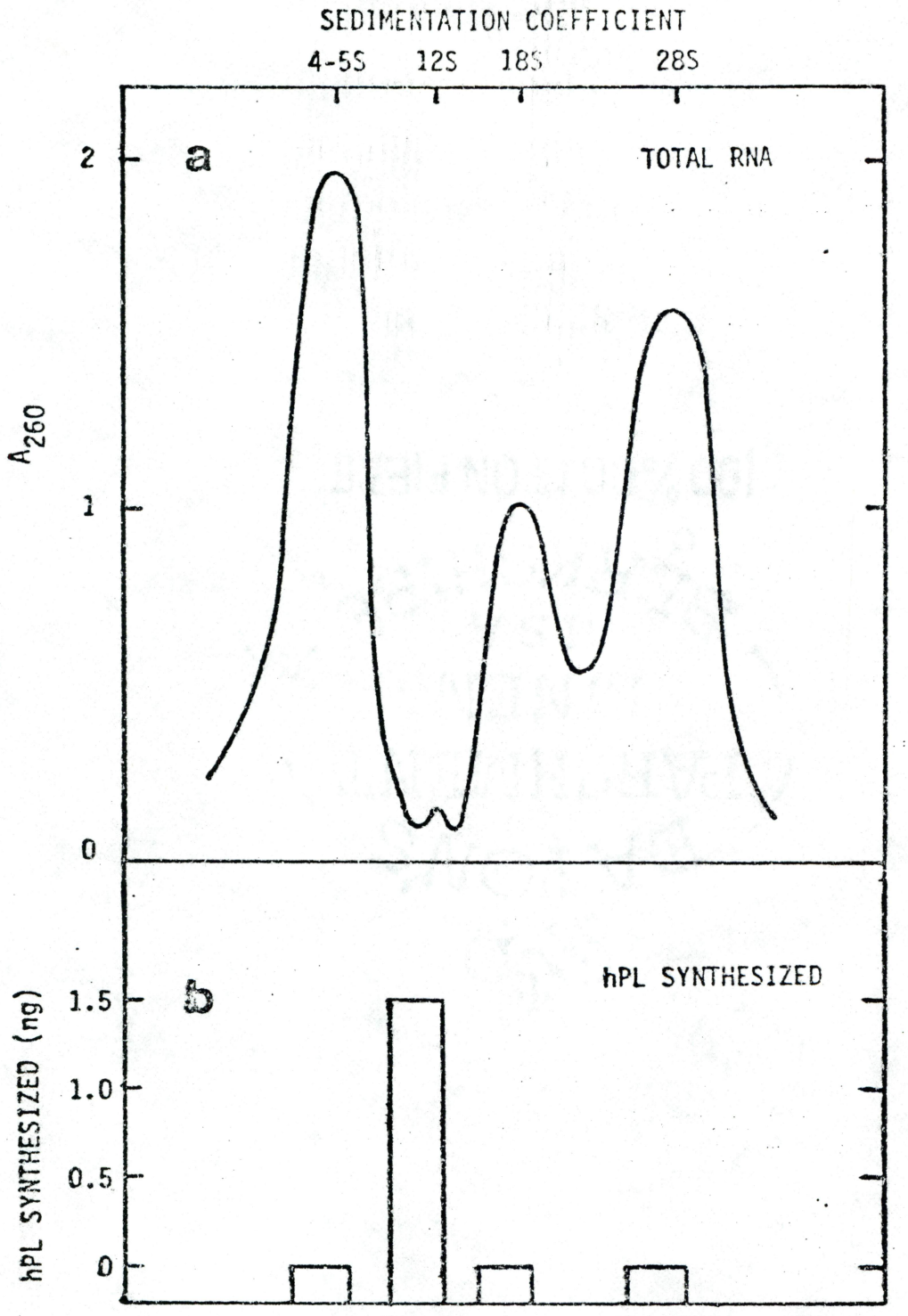

Fig. 3. Total RNA and the hPL synthesized by each fraction. (a) UV (260 nm) absorbance profile of total RNA fractionated by sucrose density centrifugation and (b) the quantity of hPL synthesized *in vitro* by equal quantities of RNA from each peak, as determined by RIA.

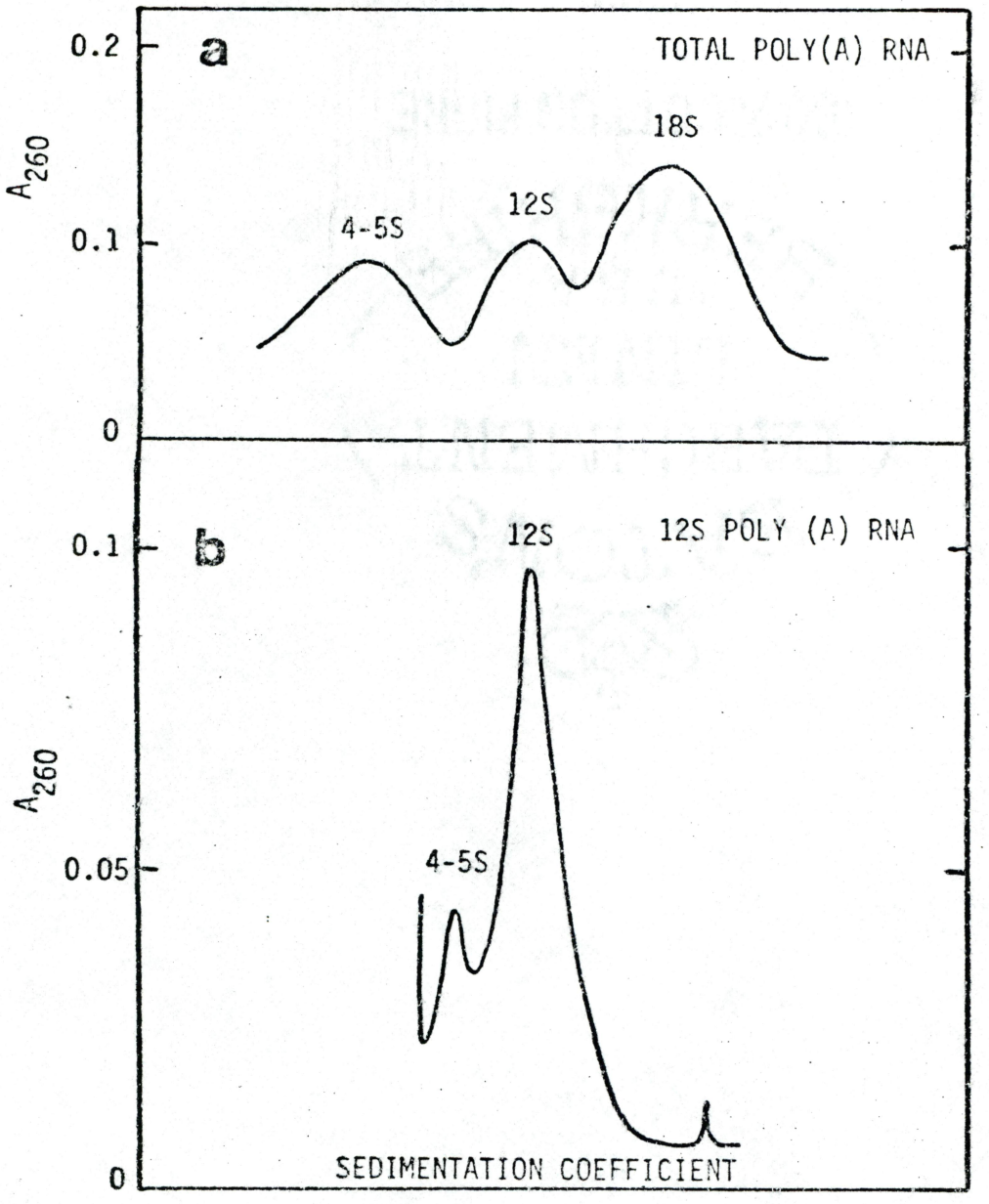

Fig. 4. Total poly (A) RNA and the isolated 12S peak. (a) UV (260 nm) absorbance profile of poly (A) mRNA isolated by oligo-(dT)-cellulose chromatography and fractionated by sucrose density centrifugation. (b) The 12S peak after isolation and centrifugation over a sucrose gradient. The 4-5S peak in each profile is carrier tRNA used in the ethanol precipitation of RNA.

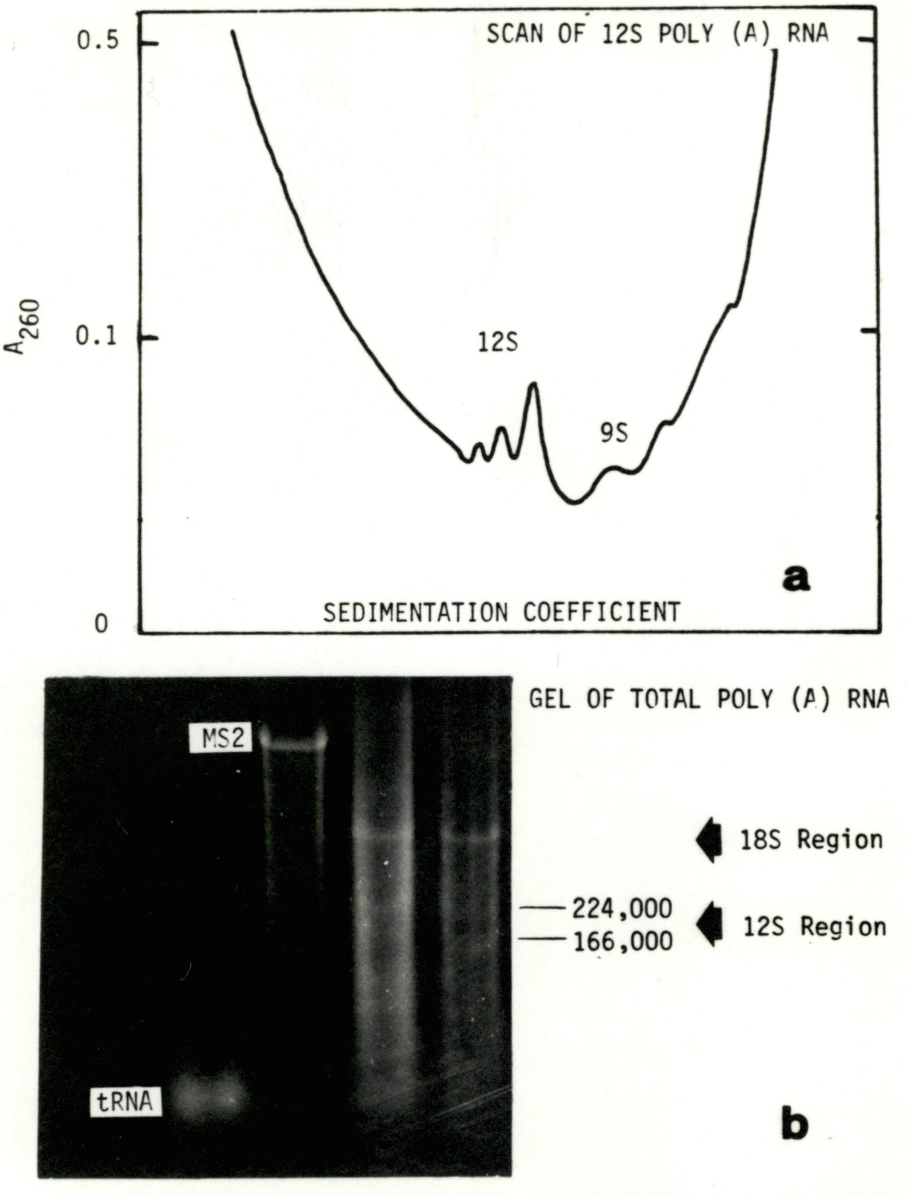

Fig. 5. The number of RNA species found in the 12S peak and their approximate molecular weights. (a) Scan of UV (260 nm) absorbance of 12S poly (A) RNA peak on a 3% acrylamide electrophoretic gel. (b) Photograph of ethidium bromide-stained total poly (A) RNA on a 1% methylmercury electrophoretic gel showing the molecular weight range of species in the 12S region. MS2 viral RNA and tRNA were used as molecular weight markers.

(Fig. 5,a) whose molecular weights ranged from approximately 166,000 to 224,000 daltons (Fig. 5,b), as determined by methylmercury gel electrophoresis.

Identification of hPL

Translation of 12S poly (A) in a cell-free system showed that as much as 24% of the total proteins synthesized could be immunoprecipitated as hPL. Reaction mixes supplemented with 12S poly (A) were found to contain 3.5 ng hPL per assay tube, as determined by RIA.

Products of translation were immunoprecipitated and subjected to electrophoresis on 12.5% Laemmli gels. Autoradiography of these gels revealed two protein bands of approximately 22,500 and 24,500 molecular weights (Fig. 6).

Identification of hCG

When assayed for beta-hCG by RIA (as for hPL), only the 12S area of the RNA profile was found to code for the subunit in the cell-free translating system (profile not shown).

Although no specific antisera were available to immunoprecipitate hCG subunits individually, autoradiograms of total proteins synthesized from 12S RNA show a definite band at about the 10,200 molecular weight range (Fig. 6).

The molecular weight of this product corresponds well with the molecular weight of the protein portion of hCG-alpha subunit (10,200 daltons). A widening of the band, corresponding to a 15,500 molecular weight endogenous protein, was also observed (Fig. 6). This may be indicative of the 16,000 molecular weight protein portion of hCG-beta subunit, since the widening is toward the heavy side of the band.

Correlations Between RNA and Synthesized Proteins

A comparison of the relative quantities of proteins synthesized by 12S poly (A) (Fig. 6) showed that the major portion of the products was in the 10,200 molecular weight range. The remaining three bands of higher molecular weights were less pronounced and probably reflected a lower concentration of message. The relative quantities of the three RNA peaks shown in Figure 5,a coincide with this observation. The concentration of each peak is consecutively greater proceeding from the higher to the lower molecular weight species. Apparently the concentration of specifically synthesized products is directly correlated with the quantity of specific mRNAs present in the 12S peak.

AUTORADIOGRAM OF SYNTHESIZED PROTEINS

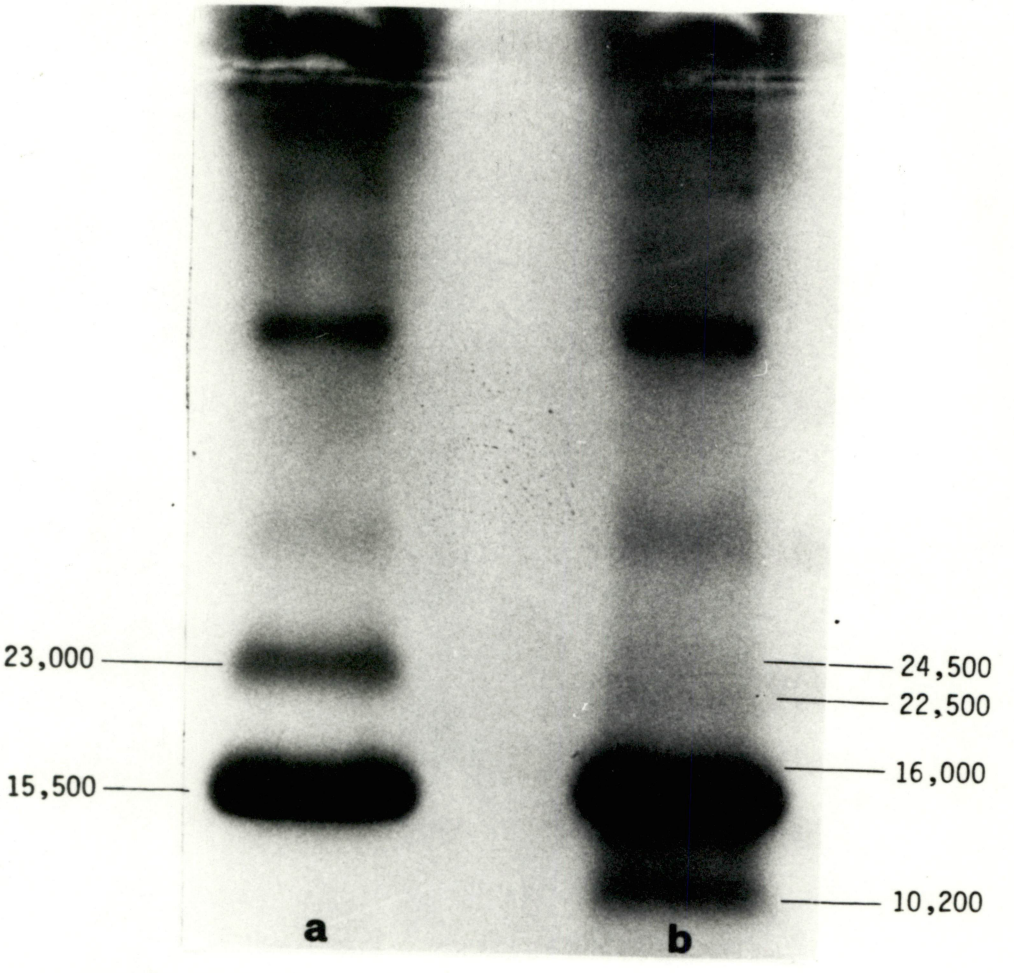

Fig 6. Autoradiogram of proteins synthesized in vitro by addition of 12S poly (A) RNA to a cell-free, rabbit reticulocyte lysate translating system. (a) Reaction mix minus RNA showing endogenous 23,000 and 15,500 molecular weight proteins. (b) Reaction mix plus 12S poly (A) RNA showing synthesized 24,500, 22,500, 10,000 and possibly 16,000 molecular weight proteins. The 23,000 molecular weight protein in (a) is lost in (b) due to competition by the addition of exogenous RNA at high concentrations.

DISCUSSION

The size estimate of 12S RNA coding for hPL made in this study is consistent with the findings of several other groups of investigators. Boime et al. (1976) have established a 12-13S mRNA for hPL while Cox et al. (1976) suggest a 13S message. A lower estimate of 11-12S has been made by Hubert and Cedard (1976). Slight differences in the interpretation of data from calibration curves could account for the range of sizes attributed to hPL mRNA. However, the range here becomes insignificant when isolating the RNA by fractionation of sucrose density gradients because technical limitations prevent a more accurate separation. Of importance, however, is that mRNA of the 12S range should be more than adequate to code for a 22,000 molecular weight protein or slightly larger precursor (Boime et al., 1976).

A size localization of hCG-beta subunit mRNA has not yet been reported in the literature, although Landfield et al. (1976) expected to find it in the 11-13S region. The 12S value reported herein (verified by RIA) becomes the first size approximation of a specific message for hCG-beta subunit. However, Landfield et al. (1976) did isolate what they consider to be the message for hCG-alpha subunit and estimated its size at 10S. If the production of the 10,200

molecular weight protein (Fig. 6) is considered suggestive of synthesis of the protein portion of hCG-alpha subunit, then it may be concluded from this study that the specific message for hCG-alpha subunit is also contained in the 12S class of placental mRNAs. Specific antisera for individual hCG subunits would be useful in the identification of the synthesized proteins. These immunologic tools would permit more definitive testing for specific products.

Whether placental mRNA codes for native hPL or for a larger pro-hormone has been a point of disagreement between investigators in this field for several years. Synthesis of a 25,000 molecular weight precursor has been reported (Boime et al., 1975; McWilliams et al., 1977; Seeburg et al., 1977) by groups using the wheat germ translation system of Roberts and Patterson (1973). A 22,000 molecular weight native hPL has been reported by Cox et al. (1976) and Hubert and Cedard (1976) using wheat germ and rabbit reticulocyte translation systems, respectively. Chatterjee et al. (1976) have also reported a 21,600 molecular weight hPL from a wheat germ system. A precise molecular weight for hPL synthesized in vitro has yet to be decided upon. This author has demonstrated both 22,500 and 24,500 molecular weight products which are immunoprecipitable as hPL. Placental 12S mRNA appears to code for a larger pro-hormone. The data also suggest that the rabbit

reticulocyte lysate system is capable of cleaving a portion of this product to yield the native hormone. Thus, autoradiography shows two immunoreactive hPL molecules of different molecular weights. That both native hormone and pro-hormone would be produced in the same assay is not an unreasonable assumption. Some membrane-independent cleavage of pro-hPL may, in fact, occur, as this system is derived from mammalian tissues.

Rabbit reticulocyte lysate is an mRNA-dependent translating system (Pelham and Jackson, 1976). Hence, the quantity of a given protein product should be proportionate to the concentration of its coding mRNA added to the system. The highest proportion of protein translated from 12S RNA is the 10,200 molecular weight band (Fig. 6) and the highest concentration of RNA in the 12S region (Fig. 5,a) is observed on the lower end of the peak (approximately 10S). Apparently, the most abundant mRNA in the 12S region codes for a 10,200 molecular weight protein which co-migrates with the hCG-alpha subunit.

It is difficult to assess the concentration of the 16,000 molecular weight protein relative to the hPL bands (Fig. 6) due to the synthesis of an endogenous 15,500 molecular weight protein. But, since a reasonably close correlation can be drawn between the sedimentation

coefficient of a particular mRNA and the molecular weight of the protein it codes for, then the middle peak in Figure 5,a should be the message for the intermediate size 16,000 molecular weight protein. Further, the concentration of this protein should be intermediate to hPL and the 10,200 molecular weight protein because of the intermediate concentration of the middle RNA peak. Finally, the largest mRNA species should be coding for the largest protein, in this case hPL. The RNA profile shows that the largest species is also the least abundant. This is unusual if one considers that hPL is the most heavily secreted protein produced by the term placenta. A factor other than mRNA concentration is suggested for the regulation of the quantity of hPL secreted by the term placenta. Similarly, one must question the fate of the 10,200 molecular weight protein which is synthesized so freely in the <u>in vitro</u> system.

SUMMARY

This investigation has demonstrated that a 12S class of RNA exists in human term placentae which contains specific messages for hPL, hCG-beta subunit and a 10,200 molecular weight protein which co-migrates with hCG-alpha subunit on SDS-acrylamide gels. Three species of RNAs are present within this 12S region, the relative concentrations and sizes of which appear to correlate well with the quantities and molecular weights of the proteins synthesized in an *in vitro* mRNA-dependent, cell-free translating system. Finally, it was shown that both native and pro-hPL are synthesized in the rabbit reticulocyte translating system.

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